

Activity of Amoxicillin-Clavulanate against Penicillin-Resistant *Streptococcus pneumoniae* in an Experimental Respiratory Infection Model in Rats†

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High doses of amoxicillin, equivalent to those produced by 500- and 750-mg oral doses in humans (area under the plasma concentration-time curve), were effective against a penicillin-resistant strain of *Streptococcus pneumoniae* in an experimental respiratory tract infection in immunocompromised rats; this superior activity confirms the results of previous studies. An unexpected enhancement of amoxicillin's antibacterial activity in vivo against penicillin-resistant and -susceptible *S. pneumoniae* strains was observed when subtherapeutic doses of amoxicillin were coadministered with the β -lactamase inhibitor potassium clavulanate. The reason for this enhancement was unclear since these organisms do not produce β -lactamase. The differential binding of clavulanic acid and amoxicillin to penicillin-binding proteins may have contributed to the observed effects.

Community-acquired respiratory tract infections caused by penicillin-resistant *Streptococcus pneumoniae* (PRSP) are becoming increasingly common worldwide (2, 13, 17), and the ideal therapy remains debatable. Oral amoxicillin and high-dose parenteral benzylpenicillin are effective against infections caused by PRSP (3, 8, 12, 13, 17), and these results have also been demonstrated with experimental models (6, 21).

The combination of amoxicillin and the β -lactamase inhibitor clavulanic acid is frequently used for the treatment of respiratory tract infections (4) and otitis media (7) because of its high level of activity against commonly implicated β -lactamase-producing pathogens, including *Haemophilus influenzae* and *Moraxella catarrhalis* (8), and penicillin-susceptible strains of *S. pneumoniae*. In experimental respiratory tract infections, oral amoxicillin-clavulanic acid demonstrated unexpected activity against strains of *S. pneumoniae* exhibiting high levels of resistance to penicillin G (22). Amoxicillin-clavulanic acid was also reported to be effective clinically against respiratory tract infections caused by PRSP (19). We therefore undertook further studies to explore the enhanced activities of β -lactam antibiotics observed against penicillin-resistant, β -lactamase-negative pneumococci when these antibiotics are coadministered with clavulanic acid.

MATERIALS AND METHODS

Bacterial strains. The MICs for and the sources of the penicillin-resistant clinical isolates of *S. pneumoniae*, isolates N1387, 11766, and 14319, and a susceptible strain of *S. pneumoniae*, strain 1629, used in these studies are presented in Table 1. Typically, PRSP strains have altered penicillin-binding proteins (PBPs) with reduced affinities for β -lactams (15). In these studies, *S. pneumoniae* N1387, the only strain whose PBP profile was characterized, displayed a PBP profile different from that of a typical penicillin-susceptible strain, *S. pneumoniae* R6, as determined by binding of ³H-benzylpenicillin (8a).

Antimicrobial agents. Amoxicillin trihydrate, ampicillin trihydrate, potassium clavulanate, and the β -lactamase inhibitor BRL 42715 (9) were prepared by SmithKline Beecham Pharmaceuticals, Worthing, United Kingdom. Amoxicillin trihydrate and ampicillin trihydrate were dissolved in 0.9% phosphate-buffered

saline (pH 8.0). The β -lactamase inhibitor BRL 42715 is an alkylidene penem that has an inhibitory profile similar to that of clavulanic acid (9).

Preparation of inoculum. For each experiment with a penicillin-resistant pneumococcus, a 1-ml stock inoculum containing 8 log₁₀ CFU/ml (frozen in aliquots at –70°C) was thawed, spread onto six nutrient agar plates containing 5% (vol/vol) defibrinated horse blood (TCS Biologicals Ltd., Buckingham, United Kingdom), and incubated overnight at 37°C. The growth from all plates was suspended in 3 ml of Todd-Hewitt (TH) broth (Oxoid), further diluted 1:5 in molten nutrient agar (Oxoid), maintained at 40°C, and used as the infective inoculum.

For each experiment with a penicillin-sensitive pneumococcus, a 16-h TH broth culture was seeded from fresh colonies grown on blood agar plates (as described above) from a 0.5-ml stock inoculum containing 7 log₁₀ CFU/ml that had been frozen at –70°C. The culture was diluted in TH broth and was used as the infective inoculum.

Preparation of animals. Male CD weanling rats (mean weight, 100 g; supplied by Charles River U.K. Ltd., Manston, United Kingdom) were used in all studies. To establish infection with the penicillin-resistant strains, rats were immunocompromised with cyclophosphamide (Endoxana; Boehringer Ingelheim, Bracknell, United Kingdom) injected intraperitoneally at 50 mg/kg of body weight 3 days prior to and on the day of infection.

Infection procedure. Before infection, the animals were anesthetized with fentanyl fluanisone (1.6 mg/kg; Hypnorm; Janssen Pharmaceuticals Ltd., Grove, United Kingdom) and diazepam (0.8 mg/kg; Valium; Roche Products Ltd., Welwyn Garden City, United Kingdom), which were separately injected intramuscularly. The animals then were infected, by nonsurgical intrabronchial instillation via intratracheal intubation (21, 22), with a 50- μ l inoculum containing approximately 6 to 7 log₁₀ CFU of *S. pneumoniae* and were allowed to recover.

Therapy. The rats received 0.5-ml oral doses of amoxicillin trihydrate or ampicillin trihydrate alone or in combination with the β -lactamase inhibitor clavulanic acid. BRL 42715 was dosed by the subcutaneous route because of its inadequate absorption from the gastrointestinal tract (9). Doses were selected to produce areas under the serum concentration-time curves (AUCs) for amoxicillin and clavulanic acid similar to those produced in human serum following the administration of standard oral doses of amoxicillin-clavulanate (Augmentin), i.e., amoxicillin-clavulanate doses of 100/50, 200/50, and 350/50 mg/kg in rats are equivalent to oral Augmentin doses of 250/125, 500/125, and 750/125 mg, respectively (Table 2) (1, 16). Therapy commenced at 24 h postinfection and continued twice daily (1000 and 2200 h) or three times daily (0900, 1600, and 2300 h) for 2 or 3 days.

Sampling and assessment. In all studies, groups of four or five rats were used and sampled at 72 or 96 h after infection (10 h after administration of the last dose). Lungs (mean lung weight, 1 g) were taken from each rat and homogenized in 1 ml of TH broth in a Colworth stomacher for 1 min. Serial dilutions were plated onto blood agar, and the plates were incubated at 37°C for bacterial enumeration.

Statistics. A two-way analysis of variance (ANOVA) was performed on the activity data for ampicillin and amoxicillin against *S. pneumoniae* N1387 to examine the overall effects of compound (ampicillin versus amoxicillin) and dose (50, 100, 200, and 400 mg/kg) and the interaction between compound and dose (i.e., whether the profiles over doses are similar for both compounds). Assuming

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TABLE 1. MICs for strains of *S. pneumoniae*^a

Origin	Strain	MIC ($\mu\text{g/ml}$)				
		Pen G	AMP	AMX	AMX/CA ^b	CA
South Africa	N1387	4	2	2	2/1	512
Hungary	11766	4	4	2	2/1	128
Hungary	14319	8	2	2	2/1	32
United Kingdom	1629	0.01	0.01	0.01	0.01/0.005	16

^a Determined by serial agar dilution with Mueller-Hinton agar with 5% (vol/vol) horse blood. Abbreviations: AMP, ampicillin; AMX, amoxicillin; CA, clavulanic acid; Pen G, penicillin G.

^b 2:1 ratio of amoxicillin:clavulanic acid was used.

a linear relationship between activity (\log_{10} CFU per gram) and dose on a logarithmic scale, regression lines were fitted for each compound. The regression analysis was used to determine the relative potency (11). The Student *t* test was applied in other studies.

RESULTS

Initially, studies were performed to determine the efficacy of oral amoxicillin and amoxicillin-clavulanate (three times daily [t.i.d.]) against respiratory infections caused by three strains of PRSP. Infections with *S. pneumoniae* 14319 and 11766 resulted in 100% mortality in untreated animals by 72 h; although untreated rats infected with N1387 survived, $6.9 \pm 1.2 \log_{10}$ CFU per pair of lungs was detectable at 72 h (Fig. 1). Amoxicillin (100 mg/kg) prevented death in all three groups, but it had little effect on the numbers of *S. pneumoniae* recovered from the lungs of infected animals at 72 h (10 h after administration of the final antibiotic dose). However, amoxicillin-clavulanate (100/50 and 200/50 mg/kg) significantly reduced numbers of all the bacterial strains recovered from the lungs at the end of the study in comparison with the numbers recovered from amoxicillin-treated rats (treated with 100 and 200 mg/kg alone) ($P < 0.05$; the Student *t* test).

Studies were undertaken to determine the lowest dose of amoxicillin that demonstrated enhanced activity against *S. pneumoniae* N1387 when amoxicillin was combined with clavulanate. Amoxicillin-clavulanate doses of 100/50 and 200/50 mg/kg significantly reduced the numbers of *S. pneumoniae* in comparison with the numbers obtained with the corresponding doses of amoxicillin alone (100 and 200 mg/kg, respectively) ($P < 0.05$) (Fig. 2). In contrast, bacterial counts in the groups treated with amoxicillin at doses of 50 and 25 mg/kg alone and in combination with clavulanate at 50 mg/kg were not significantly different from those in the untreated control group (Fig. 2). These results confirm that the potentiation of amoxicillin activity by clavulanate (t.i.d.) was evident only when amoxicillin had some effect in reducing the numbers of *S. pneumoniae* in infected lungs.

Dose-ranging studies were performed to investigate the ef-

TABLE 2. Comparison of AUCs for amoxicillin-clavulanic acid for humans and rats^a

Reference	Human		Rat	
	Dose (mg)	AUC ($\mu\text{g} \cdot \text{h/ml}$)	Dose (mg/kg)	AUC ($\mu\text{g} \cdot \text{h/ml}$)
Jackson et al. (16)	250/125	13.5/7.8	100/50	12.6/4.9
	500/125	22.5/5.9	200/50	24.4/4.7
Adam et al. (1)	500/125	20.4/8.0		

^a Values for dose and AUC are expressed as those for amoxicillin/clavulanic acid.

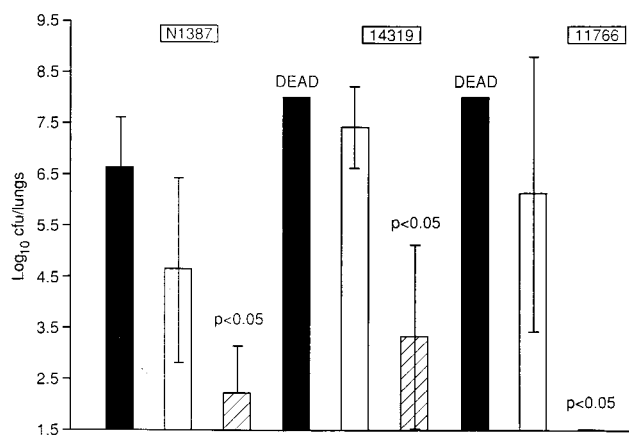


FIG. 1. Mean bacterial counts at 72 h postinfection for rats infected with one of three strains of *S. pneumoniae*. Results for each group are given as means \pm standard deviations for five rats. All compounds were administered t.i.d. Solid bars, untreated control; open bars, amoxicillin orally administered at 100 mg/kg; striped bars, amoxicillin-clavulanic acid administered orally at 100/50 mg/kg.

fects of doses of amoxicillin and amoxicillin-clavulanate following twice-daily (b.i.d.) or t.i.d. therapy and to investigate the efficacy of clavulanate alone against a respiratory infection caused by *S. pneumoniae* N1387. The relative efficacies of amoxicillin, clavulanate, and amoxicillin-clavulanate following b.i.d. therapy are presented in Table 3 (values for t.i.d. dosing are not reported). Unlike the studies described above, samples were removed later, at 96 h after infection, and in this study, the untreated group had a 100% mortality rate; interestingly, all rats treated with clavulanate alone survived the infection, although $7.9 \pm 0.5 \log_{10}$ CFU per pair of lungs persisted at 96 h. Amoxicillin at 100 mg/kg (equivalent in terms of AUC to a 250-mg dose in humans) did not significantly reduce the bacterial numbers, although it did prevent mortality. However, the coadministration of amoxicillin at 100 mg/kg and clavulanate had a significantly enhanced efficacy compared with the

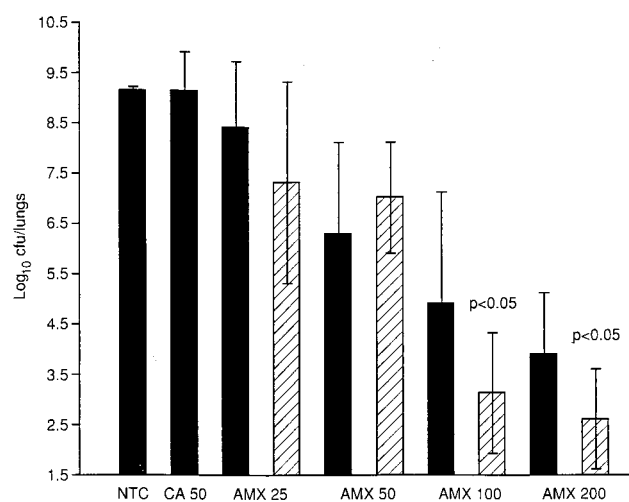


FIG. 2. Mean bacterial counts at 72 h postinfection for rats infected with *S. pneumoniae* N1387. Results for each group are given as means \pm standard deviations for five rats. All compounds were administered t.i.d. Solid bars, single treatment; striped bars, treatment plus clavulanate administered orally at 50 mg/kg. Abbreviations: AMX, amoxicillin; CA, clavulanic acid; NTC, untreated control. The numbers next to the abbreviations indicate doses (in milligrams per kilogram).

TABLE 3. Bacterial counts in the lungs at 96 h postinfection with *S. pneumoniae* N1387^a

Treatment group (dose [mg/kg]) (n = 5)	Log ₁₀ CFU/pair of lungs (mean ± SD)
Untreated control.....	No survivors
Clavulanate (50).....	7.9 ± 0.5
Amoxicillin (100).....	7.0 ± 0.5
Amoxicillin-clavulanate (100/50).....	4.1 ± 2.3 ^b
Amoxicillin (200).....	4.0 ± 2.9
Amoxicillin-clavulanate (200/50).....	2.0 ± 1.0
Amoxicillin (350).....	4.5 ± 1.8
Amoxicillin-clavulanate (350/50).....	2.3 ± 1.4

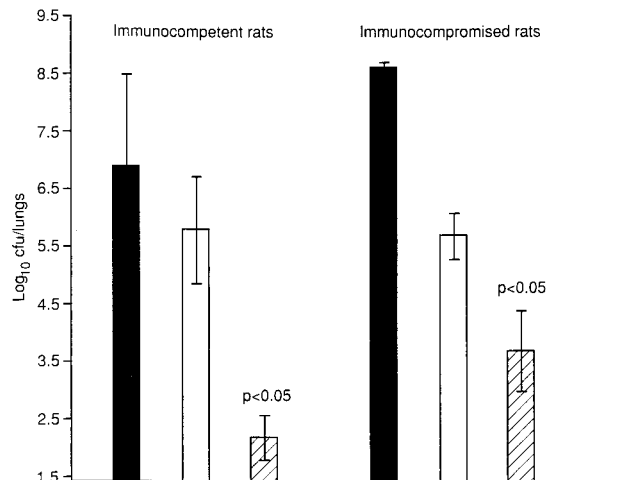
^a The compounds were administered b.i.d.^b *P* < 0.05 versus the results for treatment with amoxicillin alone.

administration of amoxicillin alone, reducing bacterial numbers by 3 log₁₀ (*P* < 0.05). Amoxicillin at doses of 200 and 350 mg/kg (equivalent to 500- and 750-mg oral doses, respectively, in humans) was highly effective in reducing the bacterial numbers, and all animals in both groups survived. Although the addition of clavulanate further reduced the bacterial numbers, the results were not significantly different from those for rats receiving either dose of amoxicillin alone (*P* > 0.05) (Table 3). Use of t.i.d. therapy for this infection (data not shown) demonstrated results similar to those obtained with b.i.d. dosing.

The comparative efficacies of a wide range of doses of amoxicillin and ampicillin against an infection caused by *S. pneumoniae* N1387 are presented in Table 4. The two-way ANOVA indicated that for a given dose the bacterial numbers remaining in the lungs after treatment with ampicillin were shown to be 1.93 log₁₀ CFU/g higher on average than those after treatment with amoxicillin, with a 95% confidence interval of 0.66 to 3.2 log₁₀ CFU/g. Overall, the activity of amoxicillin was significantly greater than that of ampicillin (*P* = 0.004). The potency of amoxicillin relative to that of ampicillin (ratio of doses required to achieve the same level of activity determined by regression analysis) was 11.4, with a 95% fiducial interval of

TABLE 4. Bacterial counts at 72 h postinfection with *S. pneumoniae* N1387^a

Study no. and treatment group (dose [mg/kg]) (n = 5)	Log ₁₀ CFU/pair of lungs (mean ± SD) ^b	
	Treatment	Treatment + clavulanate
Study 1		
Untreated control	9.2 ± 1.1	
Amoxicillin (50)	4.8 ± 1.3	
Amoxicillin (100)	5.1 ± 1.6	
Amoxicillin (200)	3.8 ± 1.0	
Amoxicillin (400)	3.3 ± 1.1	
Ampicillin (50)	6.5 ± 1.5	
Ampicillin (100)	7.2 ± 2.1	
Ampicillin (200)	5.7 ± 2.4	
Ampicillin (400)	5.2 ± 2.4	
Study 2		
Untreated control	7.0 ± 0.8	
Amoxicillin (100)	5.6 ± 0.6	3.1 ± 0.6 ^c
Ampicillin (200)	8.2 ± 1.4	8.2 ± 2.1

^a The compounds were administered t.i.d.^b Amoxicillin was significantly more effective than ampicillin overall and at each dose (*P* = 0.004; two-way ANOVA).^c *P* < 0.05 versus the results for treatment with amoxicillin alone (Student *t* test).FIG. 3. Mean bacterial counts at 72 h postinfection for rats infected with *S. pneumoniae* 1629. Results for each group are given as means ± standard deviations for five rats. All compounds were administered t.i.d. Solid bars, untreated control; open bars, amoxicillin administered orally at 10 mg/kg; striped bars, amoxicillin-clavulanate administered orally at 10-50 mg/kg.

2.1 to infinity, values which were greater than the approximate twofold difference between the two compounds in terms of oral bioavailability in favor of amoxicillin (18). In the second study, amoxicillin-clavulanate at 100/50 mg/kg was significantly more effective than amoxicillin alone at 100 mg/kg (*P* < 0.05) (Table 4), whereas ampicillin at 200 mg/kg was less effective than amoxicillin, and the addition of clavulanate to ampicillin did not enhance the activity compared to that of ampicillin alone.

The results of studies comparing the efficacies of amoxicillin alone and in combination with clavulanate against a respiratory infection with a susceptible strain of *S. pneumoniae*, strain 1629, are illustrated in Fig. 3. With a subtherapeutic dose of amoxicillin (10 mg/kg), clavulanate at 50 mg/kg significantly (*P* < 0.05) enhanced the efficacy of amoxicillin in both immunocompetent and immunocompromised rats. Clavulanate was not tested as a control in these studies since previous work demonstrated that it is not effective in reducing bacterial counts in lungs infected with either susceptible or resistant strains of pneumococci (24).

In order to exclude the possibility that clavulanate acts merely as a β -lactamase inhibitor against endogenous β -lactamase-producing microflora in the lung, an alternative β -lactamase inhibitor, BRL 42715 (50 mg/kg) (9), was tested in combination with amoxicillin (100 mg/kg) against an infection caused by *S. pneumoniae* N1387. The activity of amoxicillin was not enhanced in the presence of BRL 42715, and the bacterial counts after the coadministration of BRL 42715 and amoxicillin were 5.1 ± 0.5 log₁₀ CFU per pair of lungs, whereas they were 3.2 ± 1.6 log₁₀ CFU per lung for amoxicillin with clavulanate and 5.6 ± 0.6 log₁₀ CFU per lung for amoxicillin alone. Standard nitrocefin tests were performed with lung tissue homogenates and blood from infected animals to determine if β -lactamase was present; none was detected in a number of studies.

DISCUSSION

In the studies reported herein, the efficacy of amoxicillin against respiratory tract infections in rats caused by penicillin-resistant pneumococci (*S. pneumoniae* N1387, 11766, and 14319)

and penicillin-susceptible pneumococci (*S. pneumoniae* 1629) was enhanced by coadministration with clavulanic acid. These experimental data were obtained with amoxicillin-clavulanate at doses equivalent in terms of AUC to standard therapeutic doses of Augmentin in humans (Table 2). The enhancement was not statistically significant, however, when the doses of amoxicillin were too low to demonstrate any activity or sufficiently high for amoxicillin to be highly effective alone. Against the penicillin-susceptible strain (*S. pneumoniae* 1629), it was apparent that treatment with cyclophosphamide per se did not influence the enhancement observed.

The MICs of amoxicillin and ampicillin for the penicillin-resistant strain of *S. pneumoniae*, strain N1387, were the same (2.0 µg/ml). Overall, however, amoxicillin was the more effective agent against this respiratory tract infection (Table 4). The relative potency of amoxicillin in vivo was more than twice that of ampicillin, which is of interest considering that the bioavailability of ampicillin is approximately half that of amoxicillin in rats and other species, including humans (18). Other pharmacokinetic parameters such as elimination half-lives would not have been of importance because they are similar for the two antibiotics in both rats and humans (5, 18). Moreover, unlike with amoxicillin, clavulanate did not enhance the efficacy of ampicillin (Table 4). The coadministration of clavulanate may not, therefore, enhance the activities of all β-lactam antibiotics against the pneumococcus. In studies (23) with cefuroxime axetil (Glaxo), cefpodoxime proxetil (Roussel), and cefixime (Lederle), clavulanate did not enhance the efficacies of these β-lactams against rat respiratory tract infections caused by *S. pneumoniae* N1387, whereas some enhancement was observed for cefprozil (cefprozil monohydrate, kindly supplied by Bristol-Myers Squibb, Hounslow, United Kingdom) and occasionally for cefaclor (Lilly). These studies require confirmation, but the overall results indicate that clavulanate can influence the outcome of experimental infections, despite the absence of β-lactamase, as was evidenced by the studies with nitrocefin and with an alternative β-lactamase inhibitor, BRL 42715, which has an inhibitory profile similar to that of clavulanic acid (9).

The observation that clavulanate reproducibly enhanced the activity of amoxicillin against penicillin-resistant pneumococci was not expected. Additional in-house studies (8b) demonstrated that this enhanced efficacy is not confined to respiratory tract infections. In an experimental peritoneal infection in immunocompetent mice, amoxicillin-clavulanate was observed to have enhanced activity against *S. pneumoniae* N1387 compared with the activity of amoxicillin alone.

Greenwood (14) suggested that, in addition to the β-lactamase inhibitory activity of clavulanic acid, the compound may enhance the activities of typical β-lactam antibiotics by virtue of binding to alternative PBPs in the bacterial cell wall. This was observed in vitro by Wise (26) for certain non-β-lactamase-producing strains of the gonococcus and members of the family *Enterobacteriaceae*. It is possible, therefore, that the observed synergy may have been due to the selective binding of amoxicillin and clavulanate to different target PBPs. In preliminary in vitro studies, no synergy was observed by conventional in vitro methodologies, such as checkerboard dilution and time-kill studies (25). However, the more recent observations by Severin et al. (20) regarding the abnormal physiological properties and altered cell wall compositions of strains of *S. pneumoniae* grown in the presence of clavulanic acid, together with the antibacterial synergy with benzylpenicillin that was observed against *S. pneumoniae*, suggest that the enhanced activities of amoxicillin and other β-lactam antibiotics against the pneumococcus may be due in part to an interaction at the

cell wall and binding of clavulanic acid to PBP 3. Clavulanic acid was only poorly antibacterial (Table 1) and would not be expected to demonstrate activity in vivo. However, in the present studies, some protection with clavulanic acid was evident because all rats survived, although there was no reduction in bacterial numbers (Table 3). In contrast, there were no survivors in the untreated group.

The lack of enhancement of the efficacy of ampicillin by clavulanic acid, despite the similar MICs of amoxicillin and ampicillin, is intriguing and implies that other factors are influencing the outcome.

Severin et al. (20) suggested that the synergistic mechanisms that they observed in vitro with clavulanic acid could contribute to antibacterial efficacy in vivo. However, under our test conditions no synergy against the strains tested was observed in vitro. This does not necessarily dismiss the hypothesis that PBP binding was an influencing factor; rather, the enhancement observed was presumably due to a number of interacting factors. For example, it has recently been shown that amoxicillin-clavulanate has a greater effect than amoxicillin alone on the uptake and intracellular killing of penicillin-resistant *S. pneumoniae* by human polymorphonuclear granulocytes (10). The full significance of these findings has yet to be determined, but it appears that clavulanic acid has antibacterial properties other than those of β-lactamase inhibition.

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